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Genetics

Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bio-blots

(nucleotide analogs/avidin/alkaline phosphatase polymers/Southern blots/dot blots)

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ABSTRACT Biotin-labeled DNA probes, prepared by nick-translation in the presence of biotinylated analogs of TTP, are hybridized to DNA or RNA immobilized on nitrocellulose filters. After removal of residual probe, the filters are incubated for 2–5 min with a preformed complex made with avidin-DH (or streptavidin) and biotinylated polymers of intestinal alkaline phosphatase. The filters are then incubated with a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium, which results in the deposition of a purple precipitate at the sites of hybridization. This procedure will detect target sequences in the 1- to 10-pg range after enzyme incubation periods of 1 hr or less. The incubation period can be extended up to 24 hr, if required, to increase the color intensity of the hybridization signal. Furthermore, at high probe concentrations (250–750 ng/ml), biotin-labeled DNA exhibits lower nonspecific binding to nitrocellulose than does radiolabeled DNA, so hybridization times required for the analysis of unique mammalian gene sequences can be decreased to 1–2 hr. This nonradiographic method of probe detection should be of general utility for genetic studies using Southern, RNA, or dot-blot hybridization protocols.

Previous reports from this laboratory described the synthesis of biotin-labeled analogs of TTP and UTP that can be enzymatically incorporated into DNA and RNA, respectively (1, 2). The resulting biotin-labeled polynucleotides exhibit reassociation kinetics similar to those of biotin-free polymers and they function effectively as hybridization probes *in situ*. Hybridization signals can be visualized by indirect immunofluorescence, immunoperoxidase, or immuno-colloidal gold techniques, after incubation with a primary antibiotin antibody, and by cytochemical methods that use complexes of avidin and biotinylated peroxidase to detect the biotin-labeled probe. Such procedures have been applied successfully to the localization of specific sequences in *Drosophila* chromosomes (3, 4), mammalian metaphase chromosomes (5, 6), cultured cells (2, 7), and formalin-fixed tissue sections (2).

However, none of the visualization methods used in these studies were able to detect sequences present at the level of one copy per mammalian cell. It was apparent, therefore, that the routine application of biotin-labeled probes in genetic analysis would require the development of more sensitive biotin-detection systems.

Here we report the synthesis of biotinylated polymers of alkaline phosphatase and the construction of complexes of avidin (or streptavidin) and enzyme polymer that are 20- to 50-fold more sensitive than immunologic or affinity reagents used previously. We also describe a rapid and sensitive procedure for visualizing biotin-labeled DNA probes after hybridization to DNA or RNA immobilized on nitrocellulose filters.

MATERIALS AND METHODS

Affinity-purified rabbit antibiotin IgG was prepared as described (4). Goat antibiotin IgG was generously provided by Enzo Biochemicals (New York). Biotinylated rabbit anti-goat IgG, biotinylated goat anti-rabbit IgG, and avidin DH–biotinylated horseradish peroxidase complex (Vectastain ABC kit) were purchased or were provided as gifts from Vector Laboratories (Burlingame, CA). Streptavidin (8) was obtained from either Bethesda Research Laboratories or Enzo Biochemicals. Calf intestinal alkaline phosphatase (catalog no. 567-752) was purchased from Boehringer Mannheim. Analogs of TTP that contained a biotin molecule linked to the C-5 position of the pyrimidine ring through linker arms 4, 11, and 16 atoms long (Bio-4-dUTP, Bio-11-dUTP, and Bio-16-dUTP) were synthesized as described (1, 2). Biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester was synthesized according to Costello *et al.* (9). Samples of this ester, Bio-11-dUTP, and Bio-16-dUTP were also provided by Stanley Kline (Enzo Biochemicals). Disuccinimidyl suberate was a product of Pierce. Restriction endonucleases were obtained from New England BioLabs. Agarose (type II), bovine serum albumin (fraction V), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) *p*-toluidine salt, cadaverine free base, 3,3′-diaminobenzidine tetrahydrochloride (DAB), ethyl aminocarbazole (EAC), herring sperm DNA (type VII), and nitro blue tetrazolium (NBT; grade III) were from Sigma. Plasmids containing human globin gene sequences were provided by Sherman Weissman (Yale University). JW101 is a 0.4-kilobase-pair cDNA α-globin clone (10) and pHβC6 is a 5.2-kilobase-pair genomic fragment containing the β-globin gene in pBR322 (11). Plasmid pMM984 (12) contains the complete 5.1-kilobase genome of the parvovirus minute virus of mice cloned into pBR322. Human placental DNA was a gift from Scott Van Arsdell (Yale University).

Polymerization and Biotinylation of Alkaline Phosphatase. Calf intestinal alkaline phosphatase was polymerized by cross-linking with disuccinimidyl suberate. The enzyme was diluted

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Abbreviations: Bio-4-dUTP, Bio-11-dUTP, or Bio-16-dUTP, analogs of TTP that contain a biotin molecule linked to the C-5 position of the pyrimidine ring through linker arms that are 4, 11, or 16 atoms long; Bio-4-DNA, Bio-11-DNA, or Bio-16-DNA, DNA probes prepared with the above analogs; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; DAB, 3,3′-diaminobenzidine; EAC, ethyl aminocarbazole; ABAP or Apoly(BAP), complexes of avidin with biotinylated alkaline phosphatase or alkaline phosphatase polymers; NaCl/Cit, standard saline citrate (0.15 M NaCl/0.015 M sodium citrate, pH 7.0).

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to 1 mg/ml in ice-cold 3 M NaCl/1 mM $MgCl_2$/0.1 mM $ZnCl_2$/30 mM triethylanolamine, pH 7.6 (NMZT buffer) in a silanized glass or plastic reaction vessel. All subsequent reactions were done at 4°C. A 5 mg/ml solution of disuccinimidyl suberate in dimethylformamide was added (10 μl per ml of enzyme solution) in two equal aliquots (≈30–60 sec apart) with gentle stirring. This solution was stirred for 20 min during which a cloudy precipitate appeared; 10-μl portions of cadaverine free base (0.1 mg/ml in NMZT buffer) were added to the reaction mixture four times at 10-min intervals. The disuccinimidyl suberate/cadaverine ratio was then made equimolar by addition of another 30 μl of cadaverine solution for each ml of reaction mixture. After stirring for an additional 10 min, 2 μl of undiluted cadaverine was added and the mixture was stirred a further 30 min. The resultant clear solution was then dialyzed extensively against NMZT buffer.

Monomeric or polymeric forms of alkaline phosphatase were allowed to react with biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester by addition of 10 μl of a 20 mg/ml solution of it (in dimethylformamide) for each mg of enzyme present in the dialysis bag. After stirring on a rotary shaker at 4°C for 2 hr, the reaction mixture was again extensively dialyzed against NMZT buffer. Sodium azide was added to a final concentration of 0.02% and the biotinylated enzyme was stored at 4°C until used.

Preparation of Complexes of Avidin With Biotinylated Alkaline Phosphatase Polymer [Apoly(BAP)]. The biotinylated enzyme polymer must be mixed with a slight excess of avidin to produce complexes capable of direct interaction with biotinylated probes hybridized on filters. The protein mixture that gave an optimal signal-to-noise ratio was determined empirically by analyzing the ability of various complexes to discriminate between avidin-DH and biotinylated goat IgG spotted on nitrocellulose. Protein ratios were further adjusted for high sensitivity and specificity against Bio-16-DNA spots on nitrocellulose with a constant avidin-DH concentration and varying amounts of biotinylated enzyme polymer.

The Apoly(BAP) complex used in the experiments reported here was constructed as follows. Avidin-DH (1.8 mg/ml) was diluted to a concentration of 7.2 μg/ml into 0.1 M Tris·HCl, pH 7.5/0.1 M NaCl/2 mM $MgCl_2$/0.05% Triton X-100 (AP 7.5 buffer). The protein was added to AP 7.5 buffer in a glass tube prerinsed with 3% bovine serum albumin in AP 7.5 buffer. Biotinylated alkaline phosphatase polymer was then added to a final concentration of 1.8 μg/ml and the mixture was incubated at room temperature for at least 10 min prior to use.

Preparation of Dot-Blots. Plasmid pMM984 DNA, either linearized by *Xho* I digestion or nick-translated with Bio-11-dUTP or Bio-16-dUTP substrate (see below), was serially diluted in 50 mM Tris·HCl, pH 7.5/0.3 M NaOH containing sheared herring sperm DNA at 1.5 mg/ml. Appropriate dilutions were neutralized on ice with 3 M HCl, and 5-μl aliquots were spotted directly on BA-85 nitrocellulose filter sheets (Schleicher & Schuell). Filters were air dried and then baked for 4 hr at 80°C.

Preparation of Southern Blots. Agarose gels were prepared on a horizontal electrophoresis apparatus and DNA samples were electrophoresed as described by Alwine *et al.* (13). After the acid depurination step of Wahl *et al.* (14), DNA was transferred to nitrocellulose sheets (BA-85 or Sartorius 11336) presaturated with 20× NaCl/Cit as described by Southern and modified by Thomas (15, 16). DNA filters were air dried, baked at 80°C for 2–4 hr, and stored at 4°C over $CaSO_4$ until hybridized.

Hybridization Probes. Probes were prepared by nick-translation essentially as described by Rigby *et al.* (17). Reaction mixtures (20–200 μl) contained dCTP, dGTP, dATP, and TTP (P-L Biochemicals) or Bio-dUTP analogs, each at 50 μM. Nick-translated DNAs were purified and characterized as described (2).

Prehybridization Treatment and Hybridization Conditions. Prehybridization and hybridization buffers (10 ml/100 cm^2 of filter) were those of Wahl *et al.* (14) except that the former lacked glycine, was 25 mM in sodium phosphate (pH 6.5), and contained sonicated herring sperm DNA at 250–500 μg/ml. Hybridization buffer was 35–50% (vol/vol) in deionized formamide and contained 250–500 μg of sonicated DNA per ml. Filters were subjected to prehybridization treatment for 2–4 hr at 42°C and were hybridized at 42°C under probe concentration and time conditions given in *Results*.

For analysis of single-copy mammalian gene sequences, hybridization was generally done to a C_0t of 0.8×10^{-2} M sec^{-1}. After the hybridization, filters were washed four times at room temperature, 2–3 min for each wash—twice with 2× NaCl/Cit containing 0.1% $NaDodSO_4$ and twice with 0.2× NaCl/Cit containing 0.1% $NaDodSO_4$. Two washes (15 min each) were done with 0.16× NaCl/Cit containing 0.1% $NaDodSO_4$ (for hybridizations in 45% formamide) at 50°C. Filters were then rinsed briefly in 2× NaCl/Cit containing 0.1% $NaDodSO_4$ at room temperature, air dried, and autoradiographed or assayed for biotin as described below.

Colorimetric Detection of Bio-DNA Probes. Dry filters were incubated at 42°C for 15 min in a 3% (wt/vol) solution of bovine serum albumin in AP 7.5 buffer, air dried, baked at 80°C for 30–60 min, and then rehydrated in the albumin solution at 42°C for 20–30 min. Filters were exposed to enzyme complexes for 5 min at room temperature; 2–5 ml of complex was used for each 100 cm^2 of filter. Filters were rapidly washed three times in 250 ml of AP 7.5 buffer and twice in 0.1 M Tris·HCl, pH 9.5/0.1 M NaCl/5 mM $MgCl_2$ (AP 9.5 buffer). When avidin-peroxidase (ABC) complexes were used, 2× NaCl/Cit was substituted for AP 7.5 and AP 9.5 buffers.

For development with avidin–biotinylated alkaline phosphatase (ABAP) or Apoly(BAP) complexes, filters were incubated at room temperature in AP 9.5 buffer containing NBT (0.33 mg/ml) and BCIP (0.17 mg/ml) (18), prepared as follows. For each 15 ml of reagent, 5 mg of NBT was suspended in 1.5 ml of AP 9.5 buffer in a microcentrifuge tube and vortexed vigorously for 1–2 min and then centrifuged briefly in a Microfuge; the supernatant was decanted into 10 ml of AP 9.5 buffer warmed to 35°C in a polypropylene tube. The residual NBT pellet was extracted twice more with 1.5 ml of AP 9.5 buffer and these supernatants were pooled with the original solution. The tube was rinsed with a final 0.5 ml of AP 9.5 buffer that was also decanted into the 15-ml NBT stock solution. BCIP (2.5 mg) was dissolved in 50 μl of *N,N*-dimethylformamide and added dropwise with gentle mixing into the NBT solution.

Filters were incubated with the substrate solution in sealed polypropylene bags (Clavies; Bel-Art Products, Pequannock, NJ), 10–15 ml of solution per 100 cm^2 of filter. (To decrease nonspecific background, color development should proceed in the dark or in subdued light.) Color development was terminated by washing filters in 10 mM Tris·HCl/1 mM EDTA, pH 7.5. Developed blots were stored dry or in heat-sealed bags containing a small amount of 20 mM Tris·HCl/5 mM EDTA, pH 9.5. Although the color intensity fades when the nitrocellulose sheet is dried, rewetting with buffer will restore color as long as the filter has been stored without prolonged exposure to strong light.

RESULTS

The general utility of biotin-labeled polynucleotides as hybridization probes depends, in large part, upon the sensitivity and speed of the probe detection method. We therefore first examined the relative efficiencies of standard immunological and affinity procedures for visualizing biotin-labeled DNA. Serial 1:2 dilutions of target (pMM984 plasmid) DNA labeled with Bio-4-dUTP, Bio-11-dUTP, or Bio-16-dUTP were mixed with a constant amount of carrier herring sperm DNA (7.5 μg) and spotted directly onto nitrocellulose strips. These strips were then incubated with various detector reagents, and the sensitivity of each method was determined. Indirect immunofluorescence, with a hand-held UV-light source used for visualization, was relatively insensitive: detection limits were near 1 ng of target DNA. Indirect immunoperoxidase methods, using either DAB or EAC as a substrate, were better but still only 150–200 pg was detected with a Bio-11-DNA target. The peroxidase-antiperoxidase assay method of Sternberger *et al.* (19) improved the sensitivity ≈2-fold over that seen with the indirect immunoperoxidase technique. With each of these immunological methods, increasing the length of the biotin linker arm from 4 to 11 atoms enhanced the detectability of the target by ≈4-fold. Bio-4-DNA was not detected at all by complexes of avidin and biotinylated horseradish peroxidase [the ABC complexes of Hsu *et al.* (20)]. However, ABC complexes revealed Bio-11-DNA and Bio-16-DNA with equal efficiencies and with a sensitivity limit of <100 pg in a simple one-step reaction. Complexes made with avidin-DH and biotinylated alkaline phosphatase were even more sensitive than ABC complexes, with detection limits between 20 and 30 pg of target DNA.

None of the above techniques were sensitive enough for the analysis of single-copy mammalian DNA sequences. We reasoned that the simplest way to increase sensitivity was to construct covalently linked enzyme polymers that, after biotinylation, could be used in conjunction with avidin (or streptavidin) to make complexes for direct probe detection. Polymers of alkaline phosphatase that retained high levels of enzymatic activity were prepared. A complex of this type, termed Apoly(BAP), when used in conjunction with a substrate mixture of NBT and BCIP, would detect 1–2 pg of target DNA with enzyme incubations of 3–4 hr, a sensitivity sufficient for analyzing unique sequences in a 7.5-μg sample of mammalian DNA.

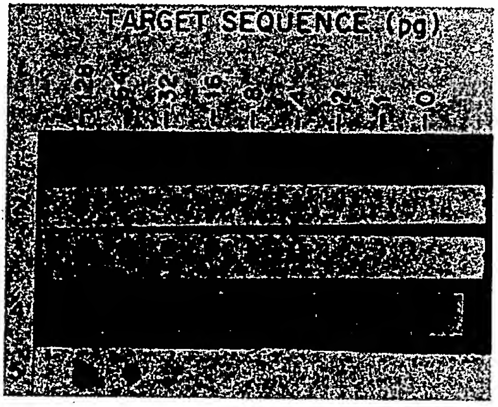

FIG. 1. Detection of Bio-DNA on nitrocellulose by avidin–biotinylated enzyme complexes. Each spot contained the indicated amount of pMM984 plasmid DNA (target sequence), labeled with Bio-16-dUTP and [α-^{32}P]dCTP (3×10^5 cpm/μg) by nick-translation, and 7.5 μg of carrier DNA. Detection systems were: lane 1, ABC (peroxidase) complex, 1-hr reaction with DAB; lane 2, ABAP complex, 3.75-hr reaction with NBT/BCIP; lane 3, Apoly(BAP) complex, 3.75-hr reaction with NBT/BCIP. Lane 4 was stained for total DNA with 0.02% toluidine blue; lane 5 is an autoradiograph of a duplicate strip exposed to film for 24 hr with screen.

Fig. 1 shows typical assay results obtained by using ABC, ABAP, and Apoly(BAP) enzyme detector complexes. Peroxidase (ABC) reactions (lane 1) generally gave higher nonspecific background on the nitrocellulose filters than did ABAP or Apoly(BAP) complexes (lanes 2 and 3, respectively), particularly if incubations with peroxidase substrates were longer than 30–60 min. However, with the Apoly(BAP) detector, 1–2 pg of target DNA was visible without significant background noise.

Previous *in situ* hybridization experiments (2, 3) indicated that biotin-labeled polynucleotides exhibited less nonspecific binding to tissues and chromosomes than did comparable radiolabeled probes. These observations suggested that high Bio-DNA probe concentrations could also be used to drive hybridization reactions on nitrocellulose. The validity of this suggestion was tested by hybridizing pMM984 DNA probes, labeled with ^{32}P alone or with both ^{32}P-labeled and biotin-labeled nucleotides, to replica dot-blot strips. The two probes of identical specific radioactivity were hybridized at probe concentrations ranging from 5 to 1,000 ng/ml (Fig. 2). The autoradiograph of the strips hybridized with ^{32}P-labeled DNA showed significant nonspecific background at all probe concentrations above 25 ng/ml (Fig. 2*C*). In contrast, the ^{32}P-labeled Bio-16-DNA probe gave virtually no nonspecific background at probe concentrations up to 750 ng/ml (Fig. 2*B*). This result demonstrated that a good signal-to-noise ratio could be obtained with high concentrations of a Bio-DNA probe, making it possible to decrease markedly the hybridization times required to achieve any desired C_0t value.

The autoradiographic signal from the ^{32}P-labeled Bio-16-DNA probe (Fig. 2*B*) was appreciably lower than that observed with the biotin-free ^{32}P-labeled probe of equivalent specific radioactivity (Fig. 2*C*). This observation, coupled with the previous report (1) that the melting temperature of biotin-labeled DNA duplexes is slightly lower than that of biotin-free DNAs, indicated that maximal specific hybridization of the Bio-16-DNA probe was not achieved under the conditions used (50% formamide buffer, 42°C). To establish better hybridization conditions, duplicate sets of filters were hybridized (and washed) at different stringencies. This study indicated that hybridization buffers containing 45% formamide, when combined with washes in 0.16× NaCl/Cit at 50°C, gave the best signal-to-noise ratios. Although decreasing the stringency further resulted in nonspecific hybridization to carrier DNA spots, there was still little nonspecific binding to the filter at a probe concentration of 500 ng/ml (data not shown).

The ability of the Apoly(BAP) detection system to visualize sequences in a Southern blot format was first analyzed in a reconstruction experiment. Various amounts of linearized pAT153 plasmid DNA were added to 10-μg samples of sheared carrier DNA and the mixtures were electrophoresed on a 1.4% agarose gel. The region of the gel containing the 3.6-kilobase plasmid bands was transferred to nitrocellulose and the filter was hybridized with a Bio-16-labeled plasmid probe. To test the detection method critically, the hybridization was done at a high stringency (in 50% formamide) to a high C_0t value, conditions known to give less than maximal results. Nevertheless, after a 2-hr incubation in the NBT/BCIP substrate solution the Apoly(BAP) complex clearly detected bands containing as little as 3.1 pg of plasmid DNA (Fig. 3). The intensity of the signal was also proportional to the amount of target sequence.

Further testing of this detector system was done by digesting human placental DNA with *Eco*RI or *Hin*dIII and transferring the resulting fragments to nitrocellulose after electrophoresis in a 1% agarose gel. The DNA was then hybridized for 2 hr with

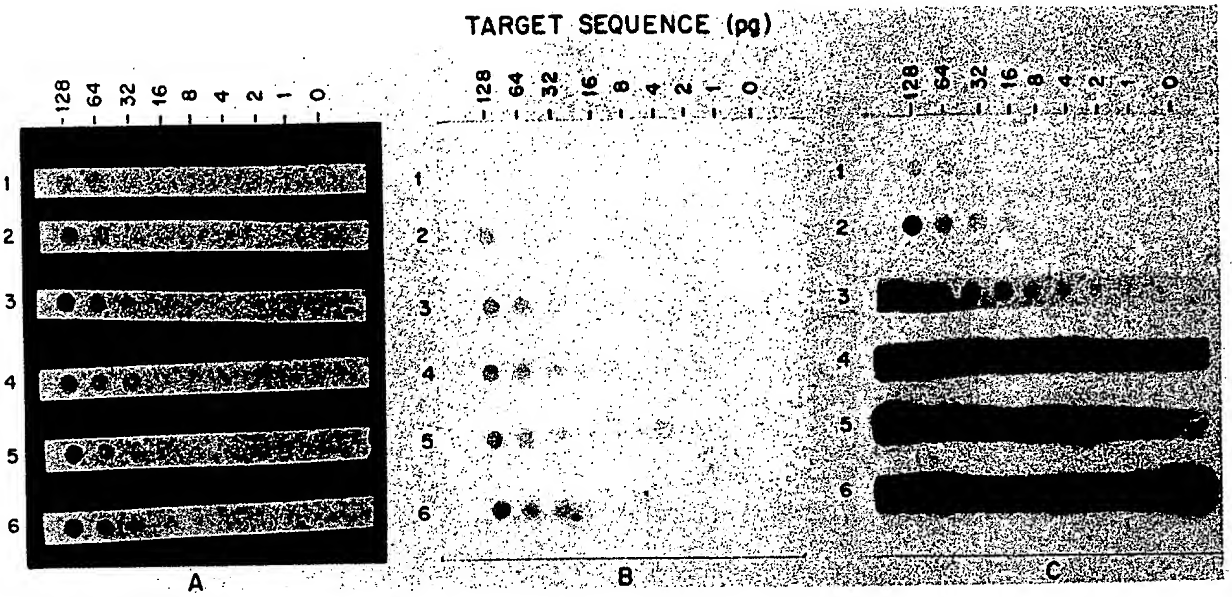

FIG. 2. Bio-DNA probes exhibit less concentration-dependent background binding to dot-blots than do biotin-free DNA probes. Each spot contained the indicated amount of unlabeled, linearized pMM984 DNA (target sequence) and 7.5 μg of carrier DNA. Dot-blots were hybridized with Bio-[^{32}P]pMM984 (*A* and *B*) or [^{32}P]pMM984 (*C*) probes (both 6.7 × 10^5 cpm/μg) for 20 hr in hybridization buffer containing 50% (vol/vol) formamide (14). Probe concentrations were: lane 1, 5 ng/ml; lane 2, 25 ng/ml; lane 3, 125 ng/ml; lane 4, 500 ng/ml; lane 5, 750 ng/ml; and lane 6, 1,000 ng/ml, respectively. (*A*) Photograph of Bio-[^{32}P]DNA probed blots after detection of Bio-DNA with Apoly(BAP) complex for 4.5 hr. (*B* and *C*) Autoradiographs of the strips after 4-day exposure to film with screen.

Bio-16-labeled α-globin (clone JW101) or Bio-16-labeled β-globin (pHβC6) probes. After the hybridized probes were visualized with the Apoly(BAP) reagent and several hours of enzyme incubation (Fig. 4), restriction fragments were observed that had sizes in good agreement with published values (21–23). The minor 2.5-kilobase *Eco*RI fragment hybridized to the β-globin probe (Fig. 4, lane 3) is most likely the *Eco*RI fragment from the 5′ region of the δ-globin gene, which cross-hybridizes with β-globin probes (23, 24). Although the three *Hin*dIII fragments that hybridized with the α-globin probe (Fig. 4, lane 2) exhibited a weaker signal than the other bands observed in lanes 1, 3, and 4, this is not surprising because each of these fragments hybridized to only a subset of the 400 nucleotides present in the probe. It is clear, however, that unique mammalian gene sequences can be visualized colorimetrically by using the Apoly(BAP) detection system. Specific RNA sequences have also been visualized on nitrocellulose by this method with similar (1–10 pg) sensitivity (data not shown). Combining Bio-DNA probes with an Apoly(BAP) detector system thus provides a rapid and sensitive procedure for Southern, RNA, or dot-blot hybridization analysis that eliminates the need for radioactive materials and autoradiography.

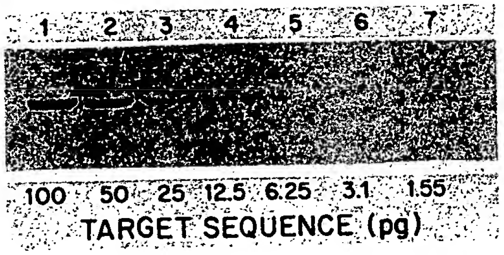

FIG. 3. Detection of complementary DNA in Southern blots by Bio-DNA hybridization and colorimetric localization is sensitive and proportional to the amount of complementary DNA. A 1.4% gel was loaded with linearized plasmid (pAT153) DNA (target sequence) in 10 μg of sheared herring sperm DNA for each lane. The appropriate region of the gel was transferred to nitrocellulose and hybridized with Bio-16-pMM984 probe (which contains all pAT153 sequences) at 0.25 μg/ml for 18 hr in 50% formamide. Bound Bio-DNA probe was detected with Apoly(BAP) complex in a 2-hr reaction.

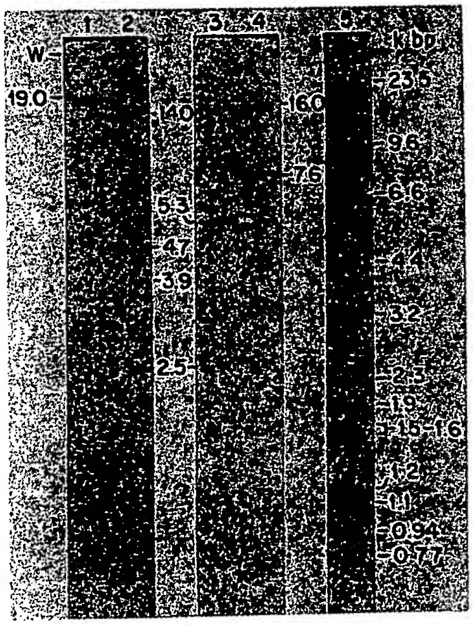

FIG. 4. Detection of α- and β-globin genes in human DNA by using Bio-DNA probes and Apoly(BAP) colorimetric visualization. *Eco*RI (lanes 1 and 3) and *Hin*dIII (lanes 2 and 4) digests of human placental DNA (15 μg per lane) were electrophoresed in a 1% agarose gel and transferred to nitrocellulose. Lanes 1 and 2 were hybridized with an α-globin probe (JW101) at 0.35 μg/ml for 2 hr in 45% formamide. Lanes 3 and 4 were hybridized with a β-globin probe (pHβC6) under the same conditions. Lane 5 contained 750 pg each of λ phage *Hin*dIII and pMM984 *Pst* I digests in 10 μg of sheared carrier DNA and was hybridized with λ and pMM984 probes. All probes were labeled with Bio-16-dUTP by nick-translation and were detected with Apoly(BAP) complexes in NBT/BCIP reactions of 1 hr (lane 5), 4 hr (lanes 3 and 4), or 9 hr (lanes 1 and 2). kbp, Kilobase pairs.

DISCUSSION

The hybridization and enzymatic detection protocol reported here offers several advantages over conventional procedures that use radioactive probes and autoradiographic detection. Because of their chemical stability, Bio-DNA probes can be used over long periods of time (at least 1–2 years) and still yield reproducible hybridization results. By using Bio-DNA probes at high concentration, the hybridization times required for the analysis of unique eukaryotic gene sequences can be reduced to 1 or 2 hr. This feature applies to studies using either colorimetric or autoradiographic detection methods. However, colorimetric visualization provides superior resolution over autoradiography with ^{32}P (Fig. 1, lanes 2 and 5). The Apoly(BAP) enzyme complex, in combination with the NBT/BCIP substrate, is sufficiently sensitive to visualize 1–2 pg of standard nick-translated probe (≈0.5 fmol of biotinylated nucleotide) after a few hours of enzyme incubation. The sensitivity of detection can be increased 4- to 5-fold simply by making the nitrocellulose filter transparent (wet with toluene), and several other procedures for signal enhancement are also available. DNA probes can be labeled with two or more biotinylated nucleotides or can be 3′-end-labeled with biotinylated nucleotides by using terminal transferase. Alternatively, the hybridization efficiency can be increased by using single-strand probes generated from sequences cloned in M13. Finally, the size of the enzyme polymer used to construct the Apoly(BAP) complex can be increased by additional chemical polymerization reactions. It is our expectation that a combination of these approaches will lead to colorimetric methods of polynucleotide visualization with femtogram sensitivity.

One limitation of the method at present is that the colored precipitate generated by the NBT/BCIP substrate mixture is extremely insoluble in all organic solvents tested. Alternative substrates for alkaline phosphatase that generate precipitates that can be solubilized are available (25); however, these substrates are not as sensitive as the NBT/BCIP mixture. With the development of more sensitive enzyme polymers or hybridization protocols, these alternative substrates could be used to reprobe the same blot.

Enzyme–polymer complexes made with avidin or streptavidin could also prove useful for antigen detection with a biotinylated antibody intermediate, for sequence analysis by the dideoxynucleotide method (26), and for visualizing clones of interest after colony (27) or plaque (28) hybridization.

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